60 Rec'd PCT/PTO 12 APR 1999

FORM PTO-1390 (REV 5-93) — U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY DOCKET NO. P564-9008
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	DATE: April 12, 1999
	U.S. APPLN. NO. (IF KNOWN, SEE 37 CFR 1.5) 09/284233

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP97/04744	1 September 1997	11 October 1996

TITLE OF INVENTION: HELICOBACTER PYLORI LIVE VACCINE

APPLICANT(S) FOR DO/EO/US: Thomas F. MEYER, Rainer HAAS, Yan ZHENGXIN, Oscar GOMEZ-DUARTE, Bernadette LUCAS

1. XX This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
   (THE BASIC FILING FEE IS ATTACHED)

2. _ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. XX This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT articles 22 and 39(1).

4. XX A proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. XX A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. XX is transmitted herewith (required only if not transmitted by the International Bureau).
   b. XX has been transmitted by the International Bureau.
   c. _ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. _ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. _ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. _ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. _ have been transmitted by the International Bureau.
   c. _ have not been made; however, the time limit for making such amendments has NOT expired.
   d. _ have not been made and will not be made.

8. _ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. _ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. _ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. _ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. _ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. XX A FIRST preliminary amendment.
    _ A SECOND or SUBSEQUENT preliminary amendment.

14. _ A substitute specification.

15. _ A change of power of attorney and/or address letter.

16. XX Other items or information: PCT/ISA/210, PCT/IPEA/416, PCT/IPEA/409, Small Entity Statement
    CHECK NO. 19260
    Drawings - 6 sheets

U.S. APPLN. NO. (IF KNOWN, SEE 37 C.F.R. 1.50)	INTERNATIONAL APPLICATION NO.: PCT/EP97/04744	ATTORNEY DOCKET NO. P564-9008 DATE: April 12, 1999

17. XX The following fees are submitted: **Basic National Fee (37 CFR 1.492(a)(1)-(5):** Search Report has been prepared by the EPO or JPO................................$840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482)...$670.00 No international preliminary examination fee paid to USPTO (37 CFR1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....................$760.00 Neither international preliminary examination fee (37 CFR 1.482) or international search fee (37 CFR 1.445(a)(2)) paid to USPTO..$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ....$ 96.00				CALCULATIONS	PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =				$840	
Surcharge of $130.00 for furnishing the oath or declaration later than _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	16 - 20 =	00	X $ 18.00	$00	
Independent Claims	02 - 3 =	00	X $ 78.00	$00	
Multiple dependent claim(s) (if applicable)			+ $260.00	$00	
TOTAL OF ABOVE CALCULATIONS =				$840	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$420	
SUBTOTAL =				$420	
Processing fee of $130.00 for furnishing the English translation later the _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$00	
TOTAL NATIONAL FEE =				$420	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$00	
TOTAL FEES ENCLOSED =				$420	
				Amount to be refunded	$
				Charged	$

a. XX A check in the amount of $420 to cover the above fees is enclosed.
b. _ Please charge my Deposit Account No. 14-1060 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.
c. XX The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1060.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Washington, D.C. 20005-5701
Telephone No. (202) 638-5000

Robert B. Murray
Reg. No. 22,980

Serial or Patent No.: ______________ Docket No.: ______

Filed or Issued: ______________

To: ______________

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN

I hereby declare that I am

( ) the owner of the small business concern identified below:
(X) an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Creatogen Biosciences GmbH

ADDRESS OF CONCERN Ulmer Straße 160a, D-86156 Augsburg, Germany

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled Helicobacter pylori live vaccine by Inventor(s) Thomas F. Meyer, Rainer Haas, Yan Zhengxin, Oscar Gomez-Duarte, Bernadette Lucas described in

(X) the specification filed herewith
( ) application serial no. ______________ filed ______________
( ) patent no. ______________, issued ______________

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME ______________

ADDRESS ______________

( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

I akcnowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Dr. Ian P. Thippleton

TITLE OF PERSON OTHER THAN OWNER CEO

ADDRESS OF PERSON SIGNING Creatogen ... Ulmer Straße 160a, ... Postfach 10 14 42, ... Augsburg Telefon (0821) 444 ...-0 Fax (0821) ...

SIGNATURE [signature] DATE March 30, 1999

Applicant or Patentee: ______________________ Attorney's
Serial or Patent No ______________________ Docket No.:
Filed or Issued: ______________________
For: ______________________

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27 (d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.
ADDRESS OF ORGANIZATION Hofgartenstraße 2, D-80539 München , Germany

TYPE OF ORGANIZATION

[ ] UNIVERISITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501 (c) (3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE ______________________)
(CITATION OF STATUTE ______________________)
[x] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c) (3) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE ______________________)
(CITATION OF STATUTE ______________________)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled Helicobacter pylori live vaccine by inventor(s) Thomas F. MEYER, Rainer HAAS, Yan ZHENGXIN, Oscar GOMEZ-DUARTE, Bernadette LUCAS described in

[X] the specification filed herewith
[ ] application serial no. ______________, filed ______________
[ ] patent no. ______________, issued ______________

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invenion is listed below * and no rights to the invention are held by any person, other than the inventor, who could not qualify as small business concern under 37 CFR 1.9 (d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9(e). *
NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME Creatogen Biosciences GmbH
ADDRESS Ulmer Straße 160a, D-86156 Augsburg, Germany
[ ] INDIVIDUAL [XX] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME ______________________
ADDRESS ______________________
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Dr. Heinrich Kuhn
TITLE IN ORGANIZATION Head of patent department
ADDRESS OF PERSON SIGNING Hofgartenstr. 8, 80539 München

SIGNATURE ______________________
Dr. Heinrich Kuhn

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Thomas F. MEYER et al

Serial No.: Unknown

Filed: April 12, 1999

For: HELICOBACTER PYLORI LIVE VACCINE

PRELIMINARY AMENDMENT

Assistant Commissioner
for Patents
Washington, D.C. 20231

April 12, 1999

Sir:

Prior to calculation of the filing fee and prior to the examination of this application, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend the claims as follows:

Claim 3, line 1, delete "or 2".

Claim 4, line 1, delete "any of claims 1-3" and insert therefor --claim 1--.

Claim 5, line 1, delete "any one of claims 1-3" and insert therefor --claim 1--.

Claim 6, line 1, delete "any one of claims 1-3 and 5" and insert therefor --claim 1--.

Claim 7, line 1, delete "any one of claims 1-6" and insert therefor --claim 1--.

Claim 10, line 1, delete "any one of claims 1-9" and insert therefor --claim 1--.

Claim 11, lines 1 and 2, delete "any one of claims 1-10" and insert therefor --claim 1--.

Claim 13, lines 2 and 3, delete "any one of claims 1-10" and insert therefor

REMARKS

The above amendment to the claims has been made to correct the multiple dependency of the claims and to put the application in better condition for examination.

In the event that any fees are due in connection with this paper, please charge our Deposit Account No. 14-1060.

Respectfully submitted,
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Robert B. Murray
Attorney for Applicants
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RBM/cb

Helicobacter pylori live vaccine

Specification

The present invention relates to novel recombinant live vaccines, which provide protective immunity against an infection by Helicobacter pylori and a method of screening H. pylori antigens for optimized vaccines.

Helicobacter is a gram-negative bacterial pathogen associated with the development of gastritis, pepic ulceration and gastric carcinoma. Several Helicobacter species colonize the stomach, most notably H. pylori, H. heilmanii and H. felis. Although H. pylori is the species most commonly associated with human infection, H. heilmanii and H. felis also have been found to infect humans. High H. pylori infection rates are observed in third world countries, as well as in industrialized countries. Among all the virulence factors described in H. pylori, urease is known to be essential for colonisation of gnobiotic pigs and nude mice. Urease is an enzyme composed of two structural subunits (UreA and UreB). Previous studies have indicated that oral immunization using recombinant UreB plus cholera toxin were able to protect mice from gastric colonisation with H. felis and H. pylori (Michetti et al., Gastroenterology 107 (1994), 1002-1011). By oral administration of recombinant UreB antigens, however, in several cases only an incomplete protection can be obtained. Other H. pylori antigens shown to give partial protection are the 87 kD vacuolar cytotoxin VacA (Cover and Blaser, J. Biol. Chem. 267 (1992), 10570; Marchetti et al., Science 267 (1995), 1655) and the 13 and 58 kD heat shock proteins HspA and HspB (Ferrero et al., Proc. Natl. Acad. Sci. USA 92 (1995), 6499).

Attenuated pathogens, e.g. bacteria, such as Salmonella, are known to be efficient live vaccines. The first indications of the efficacy of attenuated Salmonella as good vaccine in hu-

mans came from studies using a chemically mutagenized Salmonella typhi Ty21a strain (Germanier and Furer, J. Infect. Dis. 141 (1975), 553-558), tested successfully in adult volunteers (Gilman et al., J. Infect. Dis. 136 (1977), 717-723) and later on in children in a large field trial in Egypt (Whadan et al., J. Infect. Dis. 145 (1982), 292-295). The orally administered Ty21a vaccine was able to protect 96% of the Egyptian children vaccinated during three years of surveillance. Since that time new attenuated Salomonella live vector vaccines have developed (Hone et al., Vaccine 9 (1991), 810-816), in which well defined mutations incorporated into the chromosome gave rise to non-virulent strains able to induce strong immune responses after oral administration (Tacket et al., Vaccine 10 (1992), 443-446 and Tacket et al., Infect. Immun. 60 (1992), 536-541). Other advantages of the live attenuated Salmonella vaccine include its safety, easy administration, long-time protection and no adverse reactions in comparison with the former inactivated wholesale typhoid vaccines (Levine et al., Typhoid Fever Vaccines. In: Plotkin S.A., Mortimer E.A. Jr. (eds.) Vaccines. Philadelphia: WB Saunders (1988), 333-361).

Mutants of S. typhimurium have been extensively used to deliver antigens because of the possibility to use mice as an animal model, which is believed to mimick S. typhi infections in humans. The attenuation of S. typhimurium most commonly used consists in site directed mutagenesis of genes affecting the synthesis of aromatic amino acids. Such strains, designated aro mutants, have a negligible pathogenicity, as demonstrated in animal models and human trials using these constructs (Hoiseth and Stocker, Nature 291 (1981), 238-239; Tacket et al. (1992), Supra). Advantage has been taken from the potent immunogenicity of live Salmonella vaccine to deliver heterologous antigens. Expression of specific antigens in attenuated Salmonella has conferred murine protection against several bacterial pathogens. The use of recombinant live vaccines, which are capable of expressing Helicobacter antigens and protecting the vaccinated animals, has not yet been described.

The use of attenuated live vaccines for the treatment of a Helicobacter infection has also not been rendered obvious. The reason therefor being that in the course of the Helicobacter infection a strong immune response against the pathogen per se is induced, which, however, does not lead to a protective immunity. Thus, it was highly surprising that a protective immune response is achieved when using recombinant attenuated bacterial cells as antigen carriers, which are capable of expressing a DNA molecule encoding a Helicobacter antigen. Apparently, recombinant attenuated bacterial cells expressing a Helicobacter antigen are capable of creating a qualitatively different immune response against the heterologous Helicobacter antigen than Helicobacter itself does against its own homologous antigen. Surprisingly, a non-protective immune response is thus transformed into an immune response protecting against Helicobacter infections. This unexpected observation renders it possible to use recombinant attenuated pathogens, e.g. bacterial cells, particularly Salmonella, as carriers for the screening of protective antigens, to apply the protective antigens identified in this manner in any vaccine against Helicobacter infections, and to use recombinant attenuated bacteria as carriers of protective antigens for the immunization against Helicobacter infections in humans and other mammals.

Thus, a subject matter of the present invention is a recombinant attenuated pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable to express said nucleic acid molecule or capable to cause the expression of said nucleic acid in a target cell. Preferably the nucleic acid molecule is a DNA molecule.

The attenuated pathogen is a microorganism strain which is able to cause infection and preferably effective immunological protection against the actual pathogen but is no longer pathogenic per se. The attenuated pathogen can be a bacterium, a

virus, a fungus or a parasite. Preferably it is a bacterium, e.g. Salmonella, such as S. typhimurium or S. typhi, Vibrio cholerae (Mekalanos et al., Nature 306 (1983), 551-557), Shigella Species such as S. flexneri (Sizemore et al., Science 270 (1995), 299-302; Mounier et al., EMBO J. 11 (1992), 1991-1999), Listeria such as L. monocytogenes (Milon and Cossart, Trends in Microbiology 3 (1995), 451-453), Escherichia coli, Streptococcus, such as S. gordonii (Medaglini et al., Proc. Natl. Acad. Sci. USA 92 (1995) 6868-6872) or Mycobacterium, such as Bacille Calmette Guerin (Flynn, Cell. Mol. Biol. 40 Suppl. 1 (1994), 31-36). More preferably the pathogen is an attenuated enterobacterium such as Vibrio cholerae, Shigella flexneri, Escherichia coli or Salmonella. Most preferably the attenuated pathogen is a Salmonella cell, e.g. a Salmonella aro mutant cell. The attenuated pathogen, however, can be a virus, e.g. an attenuated vaccinia virus, adenovirus or pox virus.

The nucleic acid molecule which is inserted into the pathogen codes for a Helicobacter antigen, preferably a H. felis, H. heilmanii or H. pylori antigen, more preferably a H. pylori antigen. The Helicobacter antigen can be a native Helicobacter polypeptide, an immunologically reactive fragment thereof, or an immunologically reactive variant of a native polypeptide or of a fragment thereof. Further, the Helicobacter antigen can be a protective carbohydrate or a peptide mimotope simulating the three-dimensional structure of a native Helicobacter antigen. Peptide mimotopes can be obtained from peptide libraries presented on the surface of bacterial cells (cf. PCT/EP96/01130). Of course, the transformed cell can also contain several DNA molecules coding for different Helicobacter antigens.

The nucleic acid molecules coding for Heliobacter antigens may be located on an extrachromosomal vector, e.g. a plasmid, and/or integrated in the cellular chromosome of the pathogen. When the pathogen is used as a vaccine, chromosomal integra-

tion usually is preferred.

Attenuated bacteria can be used to transcribe and translate said nucleic acid molecule directly in the bacterial cell or to deliver said nucleic acid molecule to the infected target cell, such that the DNA molecule is transcribed and/or translated by the eukaryotic target cell machinery. This indirect bacterial vaccination procedure, termed here as genetic vaccination, has been successfully used with Shigella as a carrier (Sizemore, D. R., Branstrom, A. A. & Sadoff, J. C. (1995) Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. Science 270:299-302).

In a preferred embodiment of the present invention the Helicobacter antigen is urease, a urease subunit or an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. In a further preferred embodiment of the present invention the Helicobacter antigen is a secretory polypeptide from Helicobacter, an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. A process for identifying Helicobacter genes coding for such secretory polypeptides, and particularly for adhesins, has been disclosed in the international patent application PCT/EP96/02544, which is incorporated herein by reference. This process comprises

a) preparing a gene bank of H. pylori DNA in a host organism containing an inducible transposon coupled to a marker of secretory activity,
b) inducing the insertion of the transposon into the H. pylori DNA and
c) conducting a selection for clones containing a secretory gene by means of the marker, and optionally further
d) conducting a retransformation of H. pylori by means of the DNA of clones containing genes having secretory activity, wherein isogenic H. pylori mutant strains are produced by means of integrating the DNA into the chromosome, and

e) conducting a selection detecting adherence-deficient H. pylori mutant strains.

Suitable examples of antigens obtainable by the above process are selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive variants or fragments thereof or peptide mimotopes thereof. The nucleic and amino acid sequences of the antigens AlpA and AlpB have been disclosed in the international patent applications PCT/EP96/02545 and PCT/-EP96/04124, which are incorporated herein by reference. Further, the nucleic and amino acid sequences of AlpB are shown in SEQ ID NO. 1 and 2, and the nucleic and amino acid sequences of AlpA in SEQ ID NO. 3 and 4.

It is also conceivable, however, that an intracellular antigen is used which can be presented on the surface, e.g. by autolytic release, and confers immunological protection.

The presentation of the Helicobacter antigens in the recombinant pathogen according to the invention can be accomplished in different ways. The antigen or the antigens can be synthesized in a constitutive, inducible or phase variable manner in the recombinant pathogen. Concerning the constitutive or inducible synthesis of the Helicobacter antigens known expression systems can be referred to, as have been described by Sambrook et al., Molecular Cloning, A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press.

Particularly preferred the antigens are presented in a phase variable expression system. Such a phase variable expression system for the production and presentation of foreign antigens in hybrid live vaccines is disclosed in EP-B-0 565 548, which is herein incorporated by reference. In such a phase variable expression system the nucleic acid molecule encoding the Helicobacter antigen is under control of an expression signal, which is substantially inactive in the pathogen, and which is capable of being activated by a spontaneous reorganization

caused by a nucleic acid, e.g. DNA reorganization mechanism in the pathogen, e.g. a specific DNA inversion process, a specific DNA deletion process, a specific DNA replication process or a specific slipped-strand-mispairing mechanism.

A recombinant cell having a phase variable expression system is capable of forming two subpopulations A and B, wherein the division into said subpopulations occurs by spontaneous reorganization in the recombinant nucleic acid, wherein said subpopulation A is capable of infection and immunologically active per se, while subpopulation B, which is regenerated from subpopulation A, produces at least one heterologous Helicobacter antigen and acts immunologically with respect to said additional antigen.

The activation of the expression signal encoding the Helicobacter antigen can be directly accomplished by nucleic acid reorganization or, alternatively, indirectly accomplished by activation of a gene encoding a protein which controls the expression of the gene encoding the Helicobacter antigen. The indirect activation represents a system which allows the production of the Helicobacter antigen via a cascade system, which can be realized e.g. in that the gene directly controlled by DNA reorganization codes for an RNA polymerase which is specific for the promoter preceding the Helicobacter gene, or a gene regulator which in another specific manner induces the expression of the Helicobacter gene. In an especially preferred embodiment of the present invention the expression signal for the gene encoding the Helicobacter antigen is a bacteriophage promoter, e.g. a T3, T7 or SP6 promoter, and the activation of the expression signal is caused by a nucleic acid reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

The phase variable expression system can be adjusted to provide a preselected expression level of the Helicobacter antigen. This can be accomplished e.g. by modifying the nucleotide

sequence of the expression signal, which is activated by the nucleic acid reorganization mechanism, and/or by inserting further genetic regulation elements.

The Helicobacter antigens can be produced in an intracellular, as well as in an extracellular manner in the pathogen according to the invention. For instance, autotransporter systems such as the IgA-protease system (cf. for instance EP-A-0 254 090) or the E. coli AIDA-1 adhesin system (Benz et al., Mol. Microbiol. 6 (1992), 1539) are suited as extracellular secretory system. Other suitable outer membrane transporter systems are the RTX-toxin transporters, e.g. the E. coli hemolysin transport system (Hess et al., Proc. Natl. Acad. Sci. USA 93 (1996), 11458-11463).

The pathogen according to the invention can contain a second heterologous nucleic acid, e.g. DNA molecule, which codes for an immunomodulatory polypeptide influencing the immune response quantitatively or qualitatively, apart from the nucleic acid molecule encoding the Helicobacter antigen. Examples of such immunomodulatory polypeptides are immune-stimulating peptides, cytokines like IL-2, IL-6 or IL-12, chemokines, toxins, such as cholera toxin B or adhesins.

The present invention also refers to a pharmaceutical composition comprising as an active agent a recombinant attenuated pathogen as described above, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants. Preferably, the composition is a living vaccine. The vaccination routes depend upon the choice of the vaccination vector. The administration may be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters such as the vaccinal vector itself, or the route of administration. Usually the dosage comprises about 10^6 to 10^{12} cells (CFU), preferably about 10^8 to 10^{10} cells (CFU) per vaccination. Administration to a mucosal surface (e.g. ocular, intranasal, oral, gastric, intestinal, rectal, vaginal or urinary tract)

or via the parenteral route (e.g. subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal) might be chosen. A method for the preparation of the living vaccine comprises formulating the attenuated pathogen in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

The pharmaceutical composition may be provided in any suitable form, e.g. a suspension in suitable liquid carrier, such as water or milk, a capsule, a tablet etc. In a preferred embodiment of the present invention the composition is a lyophilized product which is suspended in a liquid carrier prior to use.

Further, the present invention refers to a method for preparing a recombinant attenuated pathogen as defined above, comprising the steps of a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein the recombinant pathogen, e.g. a transformed bacterial cell, is obtained, which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell and b) cultivating said recombinant attenuated pathogen under suitable conditions. If the pathogen is a bacterial cell, the nucleic acid molecule encoding the Helicobacter antigen can be located on an extrachromosomal plasmid. It is, however, also possible to insert the nucleic acid molecule into the chromosome of the pathogen.

Furthermore, the present invention refers to a method for identifying Helicobacter antigens which raise a protective immune response in a mammalian host, comprising the steps of: a) providing an expression gene bank of Helicobacter in an attenuated pathogen and b) screening the clones of the gene bank for the ability to confer a protective immunity against a Helicobacter infection in a mammalian host. Preferably, this identification process takes place in a phase variable expression system, rendering possible a stable expression of all of

the Helicobacter antigens. Recombinant clones can then be applied as "pools" for the oral immunization of test animals, such as mice. The potential of these clones as protective antigen is then determined via a challenge infection with Helicobacter, e.g. a mouse-adapted H. pylori strain. Thus, there is a possibility of directly selecting optimized H. pylori vaccine antigens.

The invention will be further illustrated by the following figures and sequence listings.

Fig. 1: shows a schematic illustration of the urease expression vector pYZ97, whereon the genes coding for the urease subunits UreA and UreB are located under transcriptional control of the T7 promoter ϕ10. There is a ribosomal binding site (RBS) between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (ori), a ß-lactamase resistance gene (bla) and 4 T7 terminators in series.

Apart from the expression by the T7 promoter, a constitutive low level expression of the urease A and B subunits can also be brought about via a cryptic promoter, which is located upstream from the T7 promoter, on the plasmid pYZ97.

Fig.2: shows the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Fig.3: shows a schematic illustration of the T7 RNA polymerase (T7RNAP) expression cassettes pYZ88, pYZ84 and pYZ114, which can be integrated into the chromosomes of bacteria.

In the high-expression cassette pYZ88 the lambda PL promoter is located in inverse orientation, upstream from the T7RNAP gene. A gene for the temperature-sensitive repressor cI 857 (cI) is under control of this promoter. A terminator of the bacteriophage fd (fdT) is situated upstream from the cI gene. The gin gene (Mertens, EMBO J. 3 (1984), 2415-2421) codes for a control enzyme of a DNA reorganization mechanism. A DNA sequence coding for the tRNA Arg is located downstream from the gin gene.

In phase A the PL promoter responsible for the expression of the T7RNAP gene is directed in the direction of the cI857 gene and the gin gene. The consequence of this is that an active repressor is formed at the permissive temperature of 28°C and reduces the transcription from the PL promoter. At a higher temperature the transcription of the PL promoter is increased, since the repressor is inactivated at least partially under such external influences. The temperature-dependent increase in the transcription also causes a corresponding increase in the expression of the following gin gene, which as a control enzyme catalyses the inversion of the PL promoter and the transition in phase B, in which the T7RNAP gene is expressed.

In the high-expression system pYZ88 a further fdT transcription terminator is located between a kanamycin-resistance gene (km) and the promoter of this gene. In this manner, the synthesis of an anti-sense RNA, inversely orientated to the T7RNAP gene, which normally contributes to the reduction of the T7RNAP expression, is reduced. This results in a high expression of the T7RNAP.

In the medium-expression system pYZ84 a transcrip-

tion terminator (fdT) is located between the PL promoter and the start of the T7RNAP gene. In this manner the expression of the T7RNAP mRNA is reduced. Additionally, the anti-sense RNA affects the T7RNAP translation. Therefore, only a medium expression occurs.

In the low-expression system pYZ114 a deletion of 100 bp in PL is additionally introduced (Δ PL). In this manner the activity of the PL promoter is reduced to a high extent, which leads to a lower T7RNAP expression and thus to a reduction of the UreA/B gene expression. In this construct the effect of the cryptic promoter on pYZ97 is already observed.

Fig.4: shows the results of an ELISA for anti-H.pylori antibodies in intestinal fluids of vaccinated mice.

Fig.5: shows the results of an ELISA for anti-H.pylori antibodies in the serum of vaccinated mice.

Fig.6: shows the urease activity in the stomach tissue of vaccinated mice after H.pyroli challenge.

SEQ ID NO. 1 and 2 show the nucleotide sequence of the adhesin gene AlpB from H. pylori and the amino acid sequence of the polypeptide coded therefrom.

SEQ ID NO. 3 and 4 show the nucleotide sequence of the adhesin gene AlpA from H. pylori and the amino acid sequence of the protein coded therefrom.

SEQ ID NO. 5 and 6 show the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Experimental part

Example 1

Cloning of the *ureA* and *ureB* genes.

The structural genes encoding the urease, *ureA* and *ureB*, have been genetically cloned from chromosomal DNA of a clinical specimen P1 (formerly 69A) isolated at the University of Amsterdam and provided by Dr. Jos van Putten. The genes were isolated by a PCR-approach using the primer pair YZ019 (5´-GGAATTCCATATGAAACTGACTCCCAAAGAG-3´) and RH132 (5´-CTGCAGTCGACTAGAAAATGCTAAAGAG-3´) for amplification. The sequence of the primers was deduced from GenBank (accession numbers M60398, X57132). The DNA sequence of primer YZ019 covered the nucleotides 2659-2679 of the published sequence and further contained a translational regulatory sequence (down stream box; Sprengart, M. L. et al., 1990, Nuc. Acid. Res. 18:1719-1723) and a cleavage site for *NdeI*. The DNA sequence of primer RH132 covered the nucleotides 5071-5088 of the published sequence and a cleavage site for *SalI*. The amplification product was 2.4 kbp in size comprising the complete coding region of *ureA* and *ureB* genes without the original transcriptional start and termination sequences from the *Helicobacter* chromosome. The purified PCR-fragment was digested with *NdeI* and *SalI* and inserted into the corresponding cloning sites of T7 expression plasmid pYZ57 to yield the plasmid pYZ97.

pYZ57 was originally derived from plasmid pT7-7, which was described by Tabor (1990, *In* Current Protocols in Molecular Biology, 16.2.1-16.2.11. Greene Publishing and Wiley-Interscience, New York). Two terminator fragments were introduced into the pT7-7 backbone at different sites by the following strategy: (1) The tandem T7 terminators. A 2.2 kbp *EcoRI/HindIII* fragment was excised from pEP12 (Brunschwig & Darzins, 1992, Gene, 111:35-41) and the purified fragment ligated with predigested pBA (Mauer, J. et al., 1997, J. Bacteriol. 179:794-804). The ligation product was digested

with *HindIII* and *ClaI*. The resulting 2.2 kbp *HindIII/ClaI* fragment was subsequently inserted into predigested pT7-7. (2) The T1 terminator. A 230 bp *HpaI/NdeI*-fragment was excised from plasmid pDS3EcoRV (provided by Dr. H. Bujard; ZMBH, Heidelberg). The fragment was then further treated with *Klenow* to generate blunt ends. The purified rrnBT1 fragment was inserted into the previous pT7-7 derivative, predigested with *BglII* and subsequently bluntended by Klenow treatment. Figure 1 describes the completed vector pYZ97 used for the expression of the urease genes coding for urease subunits UreA and UreB in *S. typhimurium*. As indicated in Figure 1, the urease genes can be controlled by the T7 promoter ϕ10. The ribosome binding site (RBS) is located between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (*ori*) and a ß-lactamase resistance gene (*bla*).

Apart from the expression controled by the T7 promoter, a constitutive moderate level expression of the urease A and B subunits does occur from a promoter driven by *Salmonella* RNA polymerase. The promoter is located upstream from the T7 promoter, on the plasmid pYZ97. For detailed molecular analysis, the purified *BglII/HindIII*-fragment of pYZ97 was subcloned into the pCR-Script™ SK(+)kit (Stratagene) and subjected to DNA-sequencing. The sequence data confirmed the various elements in their completeness (see Figure 2 and SEQ ID NO.5 and 6): part of the *ureA* gene, the down-stream box, the RBS, the T7 promoter and the T1 terminator (rrnBT1). The sequence analysis also disclosed the region between the T1 terminator region and the T7 promoter where the *Salmonella* RNA polymerase promoter is localised. The sequence data suggests a location of this constitutive promoter between nucleotides 222 - 245 which have been deduced from structural predictions (Lisser & Margalit, 1993, Nuc. Acid. Res. 21:1507-1516).

Example 2

Immunological protection by administration of live vaccine

Materials and Methods

Bacterial strains: S. typhimurium SL3261 live vector vaccine strain was used as a recipient for the recombinant H. pylori urease plasmid constructs. S. typhimurium SL3261 is an aroA transposon mutant derived from S. typhimurium SL1344 wild type strain. S. typhimurium SL3261 is a non-virulent strain that gives protection to mice against infection with wild type S. typhimurium after oral administration (Hoiseth and Stocker (1981) Supra). S. typhimurium SL3261 and derivatives thereof, which contain the urease expression plasmid pYZ97 (extrachromosomal) and the T7RNAP expression cassettes pYZ88, pYZ84 or pYZ114, respectively (integrated into the chromosome) are indicated in table 1. Luria broth or agar was used for bacterial growth at 28°C. H. pylori wild type strain grown at 37°C on serum plates was used for the challenge experiments.

Immunization of mice: Four weeks Balb/c mice purchased from Interfauna (Tuttlingen, Germany) were adapted two weeks in an animal facility before being used for experimentation. 150 μl of blood was taken retroorbitally from all mice to obtain preimmune serum. Retroorbital bleedings were repeated from all immunized mice 1 week and 3 weeks after immunization.

Eight groups of 5 mice including controls were used in this study (table 2). Group A, the naive control group, was not immunized with Salmonella neither challenged with wild type H. pylori. The rest of the groups were all orally immunized. Group B, a negative control group, did not receive Salmonella and was challenged with H. pylori. Mice from groups C to G were immunized with Salmonella vaccine strains and challenged with H. pylori. The last group H received recombinant urease B in combination with cholera toxin and was also challenged.

Prior to immunizations mice were left overnight without solid food and 4 hours without water. 100 μl of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Then mice from group B received 100 μl PBS and mice from groups C to G received 1.0 x 10^{10} CFU

of Salmonella in a 100 μl volume. Mice from group H received four times 100 μl of a mixture of recombinant H. pylori UreaseB plus cholera toxin, one dose every week. After every immunization water and food were returned to the mice.

H. pylori challenge: Four weeks after the first oral immunization mice from groups B to H were challenged with H.pylori. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 μl of 3% sodium bicarbonate were given orally to the mice using a stainless steel catheter tube, followed by an oral dose of 5.0 x 10^9 CFU/ml of Helicobacter pylori. Water and food were returned to the mice after the challenge.

Collection of blood and tissues from mice: Twelve weeks after the first immunization the mice were left overnight without food and subsequently sacrificed for analysis of protection and immune response. The mice were anaesthetized with Metoxyfluorane for terminal cardiac bleeding and prior to sacrifice by cervical dislocation. Under aseptic conditions, spleen and stomach were carefully removed from each mouse and placed on ice in separate sterile containers until further processing. Large and small intestine were obtained for further isolation of the intestinal fluid.

Processing of stomach and measurement of urease activity: The degree of H. pylori colonisation in the mouse stomach was measured by the presence of active urease in the tissue. The Jatrox-test (Röhm-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers' directions. Stomach mucosa was exposed and washed with PBS, half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing the substrate for measurement of urease activity. Absorbance at 550 nm was measured after tubes were incubated for 4 hours at room temperature. The rest of the stomach tissue was stored at -20°C for further treatments. The urease activity values obtained from the stomach of naive mice, which

did not undergo immunization or challenge, were used to create a base line to indicate the absence of H. pylori infection and therefore protection.

Table 1

UreA and UreB expressing S. typhimurium vaccine strains

Strains	Urease Expres-sion	Source
S. typhimurium SL3261	Negative	Hoiseth and Stocker
S. typhimurium SL3262 pYZ97	Constitutive Low	this study
S. typhimurium SL3261::pYZ88pYZ97	High T7-induced expression	this study
S. typhimurium SL3261::pYZ84pYZ97	Medium T7-indu-ced expression	this study
S. typhimurium SL3261::pYZ114pYZ97	Low T7-induced expression	this study

Table 2

Mice groups used for immunization

Group	Immunogen	No. of oral immunizations
A	None	0
B	PBS oral immunization	1
C	S. typhimurium S3261	1
D	S. typhimurium S3261 pYZ97	1
E	S. typhimurium S3261::pYZ88pYZ97	1
F	S. typhimurium S3261::pYZ84pYZ97	1
G	S. typhimurium S3261::pYZ114pYZ97	1
H	Urease B plus cholera toxin	4

Results:

In the control mice (groups B and C) 100% infection with H. pylori was observed. In the mice immunized with recombinant attenuated pathogens (groups D, E, F, G) between 0% and 60% infection (100% to 40% protection) was observed. Immuno-protection did not correlate with humoral anti-UreA and UreB response, suggesting that, in addition to humoral immunity, cellular immunity is critical for protection against H. pylori infection. The results indicate that oral immunization of mice with UreA and UreB delivered by S. typhimurium attenuated strain is effective to induce high levels of protection against H. pylori colonisation.

In the mice immunized with recombinant urease B plus cholera toxin considerably higher levels of urease activity were ob-

served under said experimental conditions than when administering the recombinant attenuated pathogens according to the invention.

The results of the urease test have been illustrated in table 3.

Table 3

Group	Mouse	$E_{550nm,\ 4h}$	$E_{4h} - E_{control}$	$E_{corr.} * 3$	Dilution
A	1	0,085	-0,022	-0,066	200µl+400µl
A	2	0,091	-0,016	-0,048	200µl+400µl
A	3	0,116	0,009	0,027	200µl+400µl
A	4	0,099	-0,008	-0,024	200µl+400µl
A	5	0,101	-0,006	-0,018	200µl+400µl
Control		0,107	0	0	200µl+400µl
B	1	0,394	0,292	0,876	200µl+400µl
B	2	0,464	0,362	1,086	200µl+400µl
B	3	0,329	0,227	0,681	200µl+400µl
B	4	0,527	0,425	1,275	200µl+400µl
B	5	0,462	0,36	1,08	200µl+400µl
Control		0,102	0	0	200µl+400µl
C	1	0,248	0,145	0,435	200µl+400µl
C	2	0,369	0,266	0,798	200µl+400µl
C	3	0,209	0,106	0,318	200µl+400µl
C	4	0,219	0,116	0,348	200µl+400µl
C	5	0,24	0,137	0,411	200µl+400µl
Control		0,103	0	0	200µl+400µl
D	1	0,143	0,002	0,004	300µl+300µl
D	2	0,156	0,015	0,03	300µl+300µl
D	3	0,142	0,001	0,002	300µl+300µl
D	4	0,114	-0,027	-0,054	300µl+300µl
D	5	0,133	-0,008	-0,016	300µl+300µl
Control		0,141	0	0	300µl+300µl
E	1	0,127	0,027	0,081	200µl+400µl
E	2	0,094	-0,006	-0,018	200µl+400µl
E	3	0,099	-0,001	-0,003	200µl+400µl
E	4	0,161	0,061	0,183	200µl+400µl
E	5	0,198	0,098	0,294	200µl+400µl
Control		0,1	0	0	200µl+400µl
F	1	0,166	0,025	0,05	300µl+300µl
F	2	0,145	0,004	0,008	300µl+300µl
F	3	0,166	0,025	0,05	300µl+300µl
F	4	0,154	0,013	0,026	300µl+300µl
F	5	0,301	0,16	0,32	300µl+300µl
Control		0,141	0	0	300µl+300µl
G	1	0,084	-0,019	-0,057	200µl+400µl
G	2	0,087	-0,016	-0,048	200µl+400µl
G	3	0,269	0,166	0,498	200µl+400µl
G	4	0,085	-0,018	-0,054	200µl+400µl
G	5	0,092	-0,011	-0,033	200µl+400µl
Control		0,103	0	0	200µl+400µl
H	1	0,638	0,531	1,593	200µl+400µl
H	2	0,282	0,175	0,525	200µl+400µl
H	3	0,141	0,034	0,102	200µl+400µl
H	4	0,135	0,028	0,084	200µl+400µl
H	5	0,171	0,064	0,192	200µl+400µl
Control		0,107	0	0	200µl+400µl

Example 3

Construction and molecular analysis of various recombinant *S. typhimurium* strains expressing ureA/ureB subunits.

Description of the S. typhimurium strains used for immunization experiments.

S. typhimurium SL3261(pYZ97) **(construct A)**: *S. typhimurium* SL3261 live vaccine vector strain was used as a recipient for the recombinant urease plasmid construct pYZ97.

S. typhimurium SL3261::YZ Series (pYZ97) **(construct B)**: These carrier strains are a derivative of *S. typhimurium* SL3261 which has been equipped with the T7 RNA polymerase (T7RNAP) expression cassettes schematically presented in Figure 3. These expression cassettes encode the gene for T7RNAP which is expressed in a 2-phase modus (ON/OFF) as disclosed in a previous invention of Yan et al. ("Two phase system for the production and presentation of foreign antigens in hybrid live vaccines", PCT/EP91/02478). The cassette can be integrated into the chromosome of bacteria and provide the cell in ON-position with optimal amount of T7RNAP for activation of T7RNAP-dependent expression plasmids such as pYZ97.

The principle of the YZ84 cassette is an invertible lambda PL promoter placed on a fragment that is inverted by the phage Mu invertase Gin (Yan & Meyer, 1996, J. Biotechnol. 44:197-201). Dependent on the orientation of the PL promoter either the gin gene (OFF-position) or the T7RNAP gene (ON-position) is expressed. The following regulatory elements have been included in YZ84: (1) The temperature-sensitive cI_{ts} lambda repressor (cI) which represses the PL promoter at 28°C and dissociates at 37°C. (2) The phage fd terminator (fdT) reduces expression of gin gene in order to achieve moderate inversion rates of the PL promoter on the invertible fragment.

The 2-phase expression system enables high expression rates of foreign antigens, such as the urease subunits A and B. It is well known that high expression rates of foreign antigens reduce viability of *Salmonella* carrier thus

diminishing immune response and consequently the protective potential. It was shown that the 2-phase system has a natural competence to improve survival of recombinant *Salmonella* which express large amounts of foreign antigen. In construct B, expression of the *ureA* and *ureB* genes is mainly under the control of the strong T7 promoter resulting in high production of the urease subunits. If the T7RNAP expression cassette is in OFF-position and no T7RNAP is present, the *ureA* and *ureB* genes are constitutively expressed in moderate range by the *Salmonella* promoter.

Analysis of ureA/B subunits produced by the various *S. typhimurium* strains used for immunization experiments.

Salmonella constructs A and B were first analyzed by SDS-polyacrylamide gels for expression of UreA and UreB. The recombinant strains were grown at 37°C in liquid Luria Broth supplemented with 100µg/ml Ampicillin starting from an over night culture. The bacteria were harvested at logarithmic growth phase by centrifugation and the cell pellet was resuspended in 10mM Tris-HCl and 10 mM EDTA, (pH 8.0) and cell-density adjusted to standard A_{590}=1.0 in all probes. The bacterial suspension was mixed with the same volume of SDS-sample buffer (Sambrook, J. et al. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and boiled for 5 min. 20 µl of suspension were loaded onto two SDS-10 % polyacrylamide gels; one of the gels was stained with Coomassie blue stain and the other was electroblotted onto a nitrocellulose membrane and further processed for immunoblotting. The nitrocellulose membrane carrying the transferred proteins was blocked for 45 min at room temperature in 10 (v/w)% non-fat milk Tris-buffer-saline (TBS) (TrisHCl 100mM, NaCl 150mM, pH 7.2). After three washes in TBS-0,05 (v/v)% Tween-20, a 1:2000 dilution of rabbit anti-UreB antibody (AK 201) in 5 (w/v)% non-fat milk-TBS was added to the strip and incubated overnight at 4°C. Serum was obtained from rabbit immunised with recombinant urease B subunit purified via affinity chromatography. The membrane was washed three times for 10 min with 0,05 (v/v)%

Tween-20 in PBS, and further incubated in 5 (w/v)% non-fat milk-TBS with goat anti-rabbit IgG antibody horse radish peroxidase conjugate for 45 min at room temperature. After three washes with 0.2 (v/v)% Tween-20 as above, the membrane was developed using the ECL kit (Amersham, Germany) following the recommendations of the suppliers.

Construct A: Proteins of 67 kDa and 30 kDa were observed in the Coomassie stained gel of the whole cell lysate of construct A (*S. typhimurium* strain SL3261(pYZ97); these sizes correlate very well with those of UreB and UreA, respectively. Such proteins were absent in the control lanes containing the *S. typhimurium* SL3261 strain. Immunoblot analysis of the same protein samples using a rabbit anti-UreB antibody confirmed the 67 kDa protein observed in the Coomassie stained gel as UreB. Expression of *ureB* from *S. typhimurium* strain SL3261(pYZ97) was also examined at different phases of growth by incubating at 37°C for 2, 6 and 11 hours, respectively. Expression of *ureB* was observed in all phases of growth including in the stationary phase; although, higher expression was observed at early phases of growth. The results obtained with strain SL3261(pYZ97) indicate that UreA and UreB proteins are non-toxic for *Salmonella* and can be expressed at 37°C at any phase of bacterial growth.

Construct B: Similar analysis were performed with construct B. The comparison of both constructs in SDS-PAGE analysis reveals that construct B is the more efficient producer whilst construct A has moderate expression of *ureA* and *ureB*. In the course of bacterial growth of construct B, the expression of *ureA* and *ureB* is constantly high over a longer time period even without antibiotic selection. This confirms the exceptional productivity of construct B in comparison to construct A.

In summary, our data indicate that UreA and UreB from *H. pylori* can be expressed in *S. typhimurium* without causing adverse effects to the bacteria, and are, therefore, suitable for animal protection experiments when delivered by *Salmonella* carriers.

Plasmid-stability

Plasmid stability is essential to assure stable expression of antigens coded by genes which have been cloned into such plasmids.

In vitro plasmid stability. The ampicillin resistance marker present on plasmid pYZ97 and absent in the plasmidless *S. typhimurium* strain SL3261 was used as an indicator of plasmid stability. *S. typhimurium* strain SL3261 was grown in LB liquid medium at 28°C for up to 100 generations as described previously (Summers, D. K. and D. J. Sherrat. 1984. Cell. 36:1097-1103). Every ten generations, the number of ampicillin resistant CFU was determined from the total number of colony forming units (CFU) of *Salmonella* by plating equal number of bacterial dilutions on plain LB-agar plates and LB-agar plates supplemented with 100 μg/ml ampicillin.

Plasmid stability in vivo. Plasmid stability in vivo was analyzed by examining total CFU and ampicillin resistant CFU from mice spleen, two and seven days after oral infection of mice with 5.0 X10^9 CFU of. *S. typhimurium* SL3261(pYZ97). Mice were orally infected with *Salmonella* as described above. Two days and seven days after infection mice were sacrificed under metoxyfluorane anesthesia, and the spleen was removed aseptically for further processing. The spleen was dissected in small pieces in a petri dish, mixed with 1 ml ice-cold dd$H_2$0, and passed several times through a 18 gauge needle to suspend the spleen cells. The cell suspension was then plated on LB-agar plates with or without 100 μg/ml ampicillin. Plates were incubated at 37°C overnight and colonies counted the next day.

Plasmid stability *in vivo* was analyzed after infecting mice with one oral dose of 5.0 x 10^9 CFU of *S. typhimurium* SL3261(pYZ97). Mice spleens were taken two and seven days after infection, and plated on LB-agar plates for examination of total CFU and ampicillin resistant CFU. 2.0 x 10^4 ampicillin resistant CFU were isolated from the spleens after 48 h (Table 4). The CFU counts decreased to 56 at 7 days after immunization, but again, all were ampicillin resistant. The

data indicate that plasmid pYZ97 is stable in *Salmonella* under *in vitro* and *in vivo* conditions and is suitable for the evaluation of urease subunits as protective antigens against mouse stomach colonization by *H. pylori*. The low recovery of *Salmonella* strain SL3261 seven days after infection confirms the attenuation of this strain which allows its safe use for delivery of urease into mice.

Table 4

Recovery of *S. typhimurium* SL3261pYZ97 strain from mouse spleens and evaluation of pYZ97 plasmid stability *in vivo*.

Time after infection	Total CFU [a]	Percentage of Amp^r CFU [b]
2 days	$2.0X10^4$	100
7 days	56	100

[a] Number of CFU of *S. typhimuriun* isolated on LB plates without antibiotics from the mouse spleens two and seven days after mice had been orally inoculated with $5.0X10^9$ CFU of *S. typhimuriun* strain SL3261(pYZ97).
[b] Percentage of ampicillin resistant CFU from the total No. of CFU isolated from mouse spleens.

Table 5

Examination of urease activity and streptomycin resistant *H.pylori* in stomach antrum from mice immunized with UreA and UreB-expressing *Salmonella*.

Mice group	No.	Urease activity[a]	CFU[b]
Naive Control Group	5	0.058 ± 0.004	0 ± 0
PBS Control Group	5	0.427 ± 0.059	$2.7X10^3 \pm 1.0X10^3$
SL3261pYZ97[c]	5	0.057 ± 0.006	62.6 ± 97.3

[a] Urease activity is a mean value ± standard deviation.
[b] Determination of CFU of the streptomicin resistant *H. pylori* P76 strain was carried out by plating a section of antrum stomach on serum plates supplemented with 200 μg/ml of streptomycin. *H. pylori* were recognized based on colony morphology, urease activity, and light microscopy examination. Values correspond to CFU ± standard deviation.
[c] Mice immunized with *S. typhimurium* SL3261(pYZ97) expressing *ureA* and *ureB from H. pylori* as described in Materials and Methods.

Example 4

Protection experiments with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model.

Description of the *Helicobacter pylori* strains used for the experiments

Urease-deficient *H. pylori* P11 strain is a derivative of P1, generated by transposon shuttle mutagenesis using the Tn*Max5* mini-transposon as disclosed in the invention of Haas et al. ("Verfahren zur Identifizierung sekretorischer Gene aus *Helicobacter pylori*"; PCT/EP96/02544). Insertion of Tn*Max5* has been mapped at the 3'-end of the ureA gene resulting in a defect expression of ureA and ureB due to transcriptional coupling of both genes.

Mouse-adapted *H. pylori* P49 strain was originally established by Dr. J. G. Fox (MIT, Boston, MA) from a feline isolate. *H. pylori* P76 strain is a streptomycin-resistant derivative of P49 generated by homologous recombination with chromosomal DNA from streptomycin-resistant *H. pylori* strain NCTC11637 as described by P. Nedenskov-Sorensen (1990, J. Infect. Dis. 161: 365-366).

All *H. pylori* strains were grown at 37°C in a microaerobic atmosphere (5% O_2, 85% N_2, and 10% CO_2) on serum plates (Odenbreit, S. et al. 1996. J. Bacteriol. 178:6960-6967) supplemented with 200 μg/ml of streptomycin when appropriate.

Prophylactic immunization experiments with mice.

Immunization experiments were carried out to test the ability of UreA and B delivered by *Salmonella* to protect mice from stomach colonization by *H. pylori*. In total, 5 independent immunisation experiments have been performed. Each experiment consisted of 5 groups each with 5 mice: (1) **naive control group** was mice neither immunized with *Salmonella* nor challenged with wild type *H. pylori* P49 or the streptomycin resistant derivative strain P76; (2) **PBS control group** was non-immunized mice that received PBS and were challenged

orally with *H. pylori*; (3) ***Salmonella* control group** was mice immunized with attenuated *S. typhimurium* SL3261 strain alone and challenged with *H. pylori*; and (5) the **vaccine group** was the mice immunized with appropriate recombinant *S. typhimurium* construct (A + B) expressing UreA and UreB and challenged with *H. pylori*.

Prior to immunizations, mice were left overnight without solid food and 4 hours without water. 100 μl of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Immediately after stomach neutralization, mice from the **PBS control group** received 100 μl PBS, and mice from the ***Salmonella* control group** and ***Salmonella* vaccine group**, received 5.0 X 10^9 CFU of *S. typhimurium* strain SL3261 and the various recombinant constructs, respectively, in a total volume of 100 μl. Water and food were returned to the mice after immunization.

Four weeks after the oral immunization, mice from the **PBS control-**, ***Salmonella* control-** and **vaccine-groups** were challenged with 1.0X10^9 CFU of *H. pylori*. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 μl of 3% sodium bicarbonate were given orally to mice using a stainless steel catheter tube, followed by an oral dose of 1.0 X 10^9 CFU/ml of *H. pylori* strains P49 or P76 . Water and food were returned to mice after challenge.

Example 5

Immunological analyses of protection experiments with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model

Collection of blood and intestinal fluid from mice for serological analyses.

Antibody responses were evaluated from all mice using serum and intestinal fluid. 150 μl blood were collected retro-orbitally before immunization and three weeks after immunization, before *Helicobacter* infection. The final bleeding was carried out 11 weeks after *Salmonella*

immunization (6 weeks after challenge infection) by terminal cardiac puncture under metoxyfluorane anesthesia. The small intestines were taken from mice at the end of experiment and processed as described before (Elson, C. O. et al 1984. J. Immunol. Meth. 67:101-108) with minor modifications. Briefly, the content of intestines was removed by passing 2 ml of 50mM EDTA pH 7.5 (Riedel) containing 0,1mg/ml Soybean trypsin inhibitor (Sigma). The volume was adjusted to 5 ml with 0.15 M NaCl. The samples were vortexed vigorously, centrifuged 10 min at 2,500 rpm (Heraeus, Germany), and supernatant supplemented with 50 μl of 100 mM phenylmethylsulfonylfluoride (PMSF) (Serva) in 95% ethanol, followed by centrifugation at 13,000rpm for 20 min at 4°C (Hermes). Supernatants were supplemented with 50 μl of 100 mM PMSF and 50 μl of 2% sodium azide (Merck) and left on ice 15 min before addition of 250 μl of 7% bovine serum albumine (Biomol). The samples were frozen at -20°C until further use.

Analysis of anti-urease antibodies in mouse sera and intestinal mucosa by ELISA.

Oral immunization with *Salmonella* is known to elicit IgA antibody responses. The IgA response against urease subunits in mice immunized with *S. typhimurium* construct A + B and in control mice was assessed by ELISA. A soluble extract of *H. pylori* P1 and its urease-deficient mutant derivative strain P11 was prepared in phosphate-buffer-saline by sonicating five times with a sonifier (Branson , Danbury, Conn.) at 5 sec intervals (35 % pulses) for 45 sec. This suspension was centrifuged at 13,000 rpm (Heraeus, Germany) for 10 min at 4°C to remove intact cells. The supernatant was used as antigen after determination of the protein content using the BioRad kit. 96-well microtiter plates (Nunc, Germany) were coated with 50 μl aliquot of 50 μg/ml of antigen in sodium carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4°C. The wells were blocked with 1.0 (w/v)% non-fat milk in Tris-buffer-saline (TBS) for 45 min at room temperature and washed three times with TBS-0.05% Tween-20. The assays, which were performed in triplicate, used 50 μl of serum or gut washing

diluted 1:100 or 1:2 respectively in 0.5 (w/v)% non-fat milk-TBS added to the wells and left overnight at 4°C. The wells were then washed three times with TBS-0.05% Tween 20, and a 1:3000 dilution of a goat anti-mouse IgA horse-radish peroxidase-conjugate (Sigma) was added to all wells and incubated overnight at 4°C. The color reaction was developed by incubation at 37°C for 30 min with an orthophenylendiamine substrate in sodium acetate buffer and hydrogen peroxide. The reaction was stopped with 10 N H_2SO_4 and the A_{492} was determined in an ELISA reader (Digiscan, Asys Hitech GmbH, Austria).

Mucosal antibodies: (Construct A) Intestinal fluid was taken from each sacrificed mouse at the end of the experiment (six weeks after the *H. pylori* challenge) and tested for the presence of anti-urease antibodies by using total cell extracts of *H. pylori* wild type (P1) and urease deficient mutant strains (P11). As shown in Fig. 4, the IgA antibody response against the wild type *H. pylori* extract was around 10-fold higher in immunized mice versus non-immunized or naive mice. The mucosal IgA antibody response against the urease-deficient *H. pylori* mutant was very low in all groups of mice indicating that most of the intestinal IgA antibody response in immunized mice was directed against urease.

Serum antibodies: (Construct A) The levels of serum IgA antibodies against a wild type and an urease-deficient *H. pylori* were examined prior to immunization, 3 weeks after immunization (before challenge) and 10 weeks after immunization (6 weeks after challenge with *H. pylori*). As shown in Fig. 5 panel A, the levels of anti-wild type *H. pylori* antibodies in mice immunized with urease-expressing *S. typhimurium* construct A were ~20-fold higher at three weeks and 34-fold higher ten weeks after immunization with respect to the pre-immune serum. The serum IgA antibody response against the urease-deficient *H. pylori* strain at 3 and 10 weeks was low in all groups of mice including the mice immunized with *Salmonella* construct A (Fig. 5, panel B), indicating that most of the IgA antibody response in immunized mice is directed against the urease subunits. Low serum

antibody responses against wild type *H. pylori* were also observed at ten weeks in non-immunized mice suggesting that the *H. pylori* challenge given three weeks earlier was enough to induce a specific antibody response in these mice. The IgA response to wild type *H. pylori* in mice immunized for three weeks with *S. typhimurium* SL3261 (*Salmonella* control group) increased moderately, which may be explained by the presence of antigens in *Salmonella* that are able to induce cross-reacting antibodies against *H. pylori*. In contrast, the antibody response against the urease-deficient *H. pylori* strain in immunized mice was as low as the antibody response of non-immunized mice (Fig 5, panel B). This result suggests that most of the antibody response observed in immunized mice was against urease. Low antibody response against the urease-negative mutant was observed in the 10 weeks sera from mice given PBS or immunized with *S. typhimurium* SL3261, suggesting that the antibody response observed is due to the specific immune response against the *H. pylori* antigens given to these mice three weeks earlier during challenge. A low antibody response against the urease-deficient *H. pylori* strain was observed at three weeks in mice immunized with *Salmonella* either expressing or not expressing urease, but was absent in the mice given PBS. This confirms the presence of cross-reacting epitopes between proteins from *Salmonella* and *H. pylori*, respectively. **(Construct B):** The serological analysis of mice immunized with the construct B series achieved similar results indicating that higher production of antigen by recombinant *Salmonella* does not significantly increase antibody response.

Analysis of anti-urease antibodies in mouse sera by immunoblotting.

Expression of UreA and UreB from *S. typhimurium* necessary for the induction of mice specific immune response against *H. pylori* was analyzed. Identification of *in vivo* expression of UreA and UreB was carried out by looking for anti-UreA and anti-UreB antibodies in serum of mice immunized with *Salmonella* construct A and control mice. *H. pylori* whole-cell

antigens were prepared from the wild type *H. pylori* strain P1. Bacteria were recovered from 3 serum plates, resuspended in PBS, and harvested by 10 min centrifugation at 5,000 g. The cell pellet was resuspended in 10mM Tris-HCl and 10 mM EDTA, (pH 8.0) and cell-density adjusted to standard A_{590}=1.0 in all probes. The bacterial suspension was mixed with same volume of SDS-sample buffer (Sambrook, 1989) and boiled for 5 min. 20 μl Pellet were loaded onto a SDS-10% polyacrylamide gel. The proteins were electro-blotted onto a nitrocellulose membrane and cut into strips which were blocked for 45 min at room temperature in 10 (v/w)% non-fat milk Tris-buffer-saline (TBS) (TrisHCl 100mM, NaCl 150mM, pH 7.2). After three washes in TBS-0,05 (v/v)% Tween-20, a 1:80 dilution of mouse serum in 5 (w/v)% non-fat milk-TBS was added to the strips and incubated overnight at 4°C. Sera was obtained from mice non-immunized and immunized with *Salmonella*. After three washes, the strips were incubated with a goat anti-mouse IgG horse-radish peroxidase conjugate (Sigma) diluted 1:3000 in 5 (w/v)% non-fat milk-TBS. The ECL chemi-luminescence detection kit (Amersham, Germany) was used for development of blots according to the supplier' s directions.

Serum from immunized and non-immunized mice was obtained 3 weeks after immunization prior to the challenge with *H. pylori* and tested against whole-cell lysates of the wild type *H. pylori* P1 strain expressing UreA and UreB. Proteins of 67 kDa and 30 kDa in size, corresponding to UreB and UreA, respectively, were recognized by serum from immunized mice immunized with construct A. These bands were not observed in strips tested with serum from non-immunized mice or mice immunized with *Salmonella* only, suggesting that urease expressed by the *Salmonella* vaccine strain was able to induce a specific antibody response against both UreA and UreB of a wild type *H. pylori* strain. Similar results were obtained with construct B.

Example 6

<u>Determination of *H. pylori* colonisation in mice pretreated with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model</u>

Processing of stomach and measurement of urease activity.

Urease-test: Analysis of protection against stomach colonization by *H. pylori* was performed by testing for urease activity in the antral portion of the mouse stomach. Measurement of urease activity is a very reliable, sensitive and specific method to test for the presence of *H. pylori* infection (NIH consensus development on *Helicobacter pylori* in peptic ulcer disease. 1994. *Helicobacter pylori* in peptic ulcer disease. JAMA. 272:65) and is routinely used in clinical settings (Kawanishi, M., S. et al 1995. J. Gastroenterol. 30:16-20; Kamija, S. et al 1993. Eur. J. Epidemiol. 9:450-452; Conti-Nibali, S. et al 1990. Am. J. Gastroenterol. 85:1573-1575) and in animal research (Gottfried, M. R. et al 1990. Am. J. Gastroenterol. 85:813-818). The Jatrox-test (Röhn-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers directions. Eleven weeks after immunization with *Salmonella*, mice were sacrificed and the stomach was carefully removed under aseptic conditions. The stomach was placed in ice-cold PBS in an sterile container, and the mucosa was exposed by making an incision along the minor curvature with a sterile blade. The stomach was rinsed with PBS to remove food residues and dissected to isolate the antral region from the corpus region. Half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing 500 μl of the urease substrate from Jatrox-test. The stomach sample was incubated 4 h at room temperature and the absorbance at 550 nm (A_{550}) measured. The urease activity values obtained from the stomach of naive mice, which did not undergo immunization or challenge, were used to determine the baseline. The baseline corresponded to the average urease activity value from five naive mice stomachs tested plus two times the standard deviation of this average. Urease activity values higher than the baseline were considered *H. pylori* colonization positive and values below the baseline were

considered *H. pylori* colonization negative.

Cultivation experiment: The left portion of the antral region of stomachs obtained from mice challenged with the streptomycin resistant *H. pylori* strain P76 were plated on serum plates supplemented with 200 μg/ml of streptomycin and incubated under standard conditions. After three days incubation, bacteria were identified as *H. pylori* based on colony morphology, microscopic examination, and urease activity. The number of colony forming units (CFU) of *H. pylori* grown on plates was determined from each mouse stomach sample.

Urease test (Construct A vs. B): Mice immunized with ~5.0X10^9 CFU of *Salmonella* and challenged with 1.0X10^9 CFU of *H. pylori* strain P49, as well as control mice, were sacrificed under anesthesia and a section of the antral region of the stomach was taken for measurement of urease activity. As shown in Fig. 6, 100% of the mice immunized with UreA and B delivered by *Salmonella* construct A had urease activity below the baseline, indicating the absence of *H. pylori* colonisation. In contrast, 100 % of the non-immunized mice (PBS) and the mice immunized with *S. typhimurium* strain SL3261 alone, had urease activity measurements far above the baseline indicating stomach colonization by *H. pylori*. The naive group of mice, which did not undergo immunization or challenge, was used to set the baseline of urease activity.

Salmonella of the construct B-series had urease activity values above the baseline indicating stomach colonization by *H. pylori* challenge strain. However, the urease activities within this group were lower as in the controls suggesting a partial protection status of mice immunized with the Salmonella construct B series (Figure 6). Both Salmonella constructs, A and B, mediate similar antibody response but differed in expression of ureA and ureB. We conclude from this that the quantity of expressed urease antigen is relevant to gain optimal protection.

Construct A: To correlate stomach colonization by *H. pylori* with urease activity a new protection experiment was

performed by immunizing mice orally with *Salmonella* construct A and challenging them with the streptomycin resistant *H. pylori* P76 strain. Urease activity values correlated with the number of CFU of *H. pylori* identified. In two of the five mice immunized with urease-expressing *Salmonella*, no *H. pylori* CFU were detected and the average number of CFU in all five immunized mice was only 62. In contrast, the number of CFU in non-immunized mice was 2,737, which corresponds to 44-fold higher colonization. These data indicate that mice immunized with urease-expressing *Salmonella* were able to eliminate or significantly decrease colonizing *H. pylori* from mouse stomachs.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Max-Planck-Gesellschaft zur Foerderung der Wissenschaften e.V. Berlin
(B) STREET: Hofgartenstr. 2
(C) CITY: Muenchen
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): 80539

(ii) TITLE OF INVENTION: Helicobacter pylori live vaccine

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1557 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helicobacter pylori

(vii) IMMEDIATE SOURCE:
(B) CLONE: alpB

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1554

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 518 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1557 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helicobacter pylori

(vii) IMMEDIATE SOURCE:
(B) CLONE: alpA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1554

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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576
Gly Asn Val Phe Phe Asn Ala Met Asn Lys Ala Leu Glu Asn Lys Asn
695 700 705 710

GGA ACT AGT AGT GCT AGT GGA ACT AGT GGT GCG ACT GGT TCA GAT GGT
624
Gly Thr Ser Ser Ala Ser Gly Thr Ser Gly Ala Thr Gly Ser Asp Gly
715 720 725

CAA ACT TAC TCC ACA CAA GCT ATC CAA TAC CTT CAA GGC CAA CAA AAT
672
Gln Thr Tyr Ser Thr Gln Ala Ile Gln Tyr Leu Gln Gly Gln Gln Asn
730 735 740

ATC TTA AAT AAC GCA GCG AAC TTG CTC AAG CAA GAT GAA TTG CTC TTA
720
Ile Leu Asn Asn Ala Ala Asn Leu Leu Lys Gln Asp Glu Leu Leu Leu
745 750 755

GAA GCT TTC AAC TCT GCC GTA GCC GCC AAC ATT GGG AAT AAG GAA TTC
768
Glu Ala Phe Asn Ser Ala Val Ala Ala Asn Ile Gly Asn Lys Glu Phe
760 765 770

AAT TCA GCC GCT TTT ACA GGT TTG GTG CAA GGC ATT ATT GAT CAA TCT
816
Asn Ser Ala Ala Phe Thr Gly Leu Val Gln Gly Ile Ile Asp Gln Ser
775 780 785 790

CAA GCG GTT TAT AAC GAG CTC ACT AAA AAC ACC ATT AGC GGG AGT GCG
864
Gln Ala Val Tyr Asn Glu Leu Thr Lys Asn Thr Ile Ser Gly Ser Ala
795 800 805

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GTT ATT AGC GCT GGG ATA AAC TCC AAC CAA GCT AAC GCT GTG CAA GGG
   912
Val Ile Ser Ala Gly Ile Asn Ser Asn Gln Ala Asn Ala Val Gln Gly
            810                 815                 820

CGC GCT AGT CAG CTC CCT AAC GCT CTT TAT AAC GCG CAA GTA ACT TTG
   960
Arg Ala Ser Gln Leu Pro Asn Ala Leu Tyr Asn Ala Gln Val Thr Leu
        825                 830                 835

GAT AAA ATC AAT GCG CTC AAT AAT CAA GTG AGA AGC ATG CCT TAC TTG
  1008
Asp Lys Ile Asn Ala Leu Asn Asn Gln Val Arg Ser Met Pro Tyr Leu
    840                 845                 850

CCC CAA TTC AGA GCC GGG AAC AGC CGT TCA ACG AAT ATT TTA AAC GGG
  1056
Pro Gln Phe Arg Ala Gly Asn Ser Arg Ser Thr Asn Ile Leu Asn Gly
855                 860                 865                 870

TTT TAC ACC AAA ATA GGC TAT AAG CAA TTC TTC GGG AAG AAA AGG AAT
  1104
Phe Tyr Thr Lys Ile Gly Tyr Lys Gln Phe Phe Gly Lys Lys Arg Asn
                875                 880                 885

ATC GGT TTG CGC TAT TAT GGT TTC TTT TCT TAT AAC GGA GCG AGC GTG
  1152
Ile Gly Leu Arg Tyr Tyr Gly Phe Phe Ser Tyr Asn Gly Ala Ser Val
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  1200
Gly Phe Arg Ser Thr Gln Asn Asn Val Gly Leu Tyr Thr Tyr Gly Val
        905                 910                 915

GGG ACT GAT GTG TTG TAT AAC ATC TTT AGC CGC TCC TAT CAA AAC CGC
  1248
Gly Thr Asp Val Leu Tyr Asn Ile Phe Ser Arg Ser Tyr Gln Asn Arg
    920                 925                 930

TCT GTG GAT ATG GGC TTT TTT AGC GGT ATC CAA TTA GCC GGT GAG ACC
  1296
Ser Val Asp Met Gly Phe Phe Ser Gly Ile Gln Leu Ala Gly Glu Thr
935                 940                 945                 950

TTC CAA TCC ACG CTC AGA GAT GAC CCC AAT GTG AAA TTG CAT GGG AAA
  1344
Phe Gln Ser Thr Leu Arg Asp Asp Pro Asn Val Lys Leu His Gly Lys
                955                 960                 965

ATC AAT AAC ACG CAC TTC CAG TTC CTC TTT GAC TTC GGT ATG AGG ATG
  1392
Ile Asn Asn Thr His Phe Gln Phe Leu Phe Asp Phe Gly Met Arg Met
            970                 975                 980
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AAC TTC GGT AAG TTG GAC GGG AAA TCC AAC CGC CAC AAC CAG CAC ACG 1440
Asn Phe Gly Lys Leu Asp Gly Lys Ser Asn Arg His Asn Gln His Thr
985 990 995

GTG GAA TTT GGC GTA GTG GTG CCT ACG ATT TAT AAC ACT TAT TAC AAA 1488
Val Glu Phe Gly Val Val Val Pro Thr Ile Tyr Asn Thr Tyr Tyr Lys
1000 1005 1010

TCA GCA GGG ACT ACC GTG AAG TAT TTC CGT CCT TAT AGC GTT TAT TGG 1536
Ser Ala Gly Thr Thr Val Lys Tyr Phe Arg Pro Tyr Ser Val Tyr Trp
1015 1020 1025 1030

TCT TAT GGG TAT TCA TTC TAA 1557
Ser Tyr Gly Tyr Ser Phe
1035

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 518 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ile Lys Lys Asn Arg Thr Leu Phe Leu Ser Leu Ala Leu Cys Ala
1 5 10 15

Ser Ile Ser Tyr Ala Glu Asp Asp Gly Gly Phe Phe Thr Val Gly Tyr
20 25 30

Gln Leu Gly Gln Val Met Gln Asp Val Gln Asn Pro Gly Gly Ala Lys
35 40 45

Ser Asp Glu Leu Ala Arg Glu Leu Asn Ala Asp Val Thr Asn Asn Ile
50 55 60

Leu Asn Asn Asn Thr Gly Gly Asn Ile Ala Gly Ala Leu Ser Asn Ala
65 70 75 80

Phe Ser Gln Tyr Leu Tyr Ser Leu Leu Gly Ala Tyr Pro Thr Lys Leu
85 90 95

Asn Gly Ser Asp Val Ser Ala Asn Ala Leu Leu Ser Gly Ala Val Gly
100 105 110

Ser Gly Thr Cys Ala Ala Ala Gly Thr Ala Gly Gly Thr Ser Leu Asn
115 120 125

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Thr Gln Ser Thr Cys Thr Val Ala Gly Tyr Tyr Trp Leu Pro Ser Leu
    130                 135                 140

Thr Asp Arg Ile Leu Ser Thr Ile Gly Ser Gln Thr Asn Tyr Gly Thr
145                 150                 155                 160

Asn Thr Asn Phe Pro Asn Met Gln Gln Gln Leu Thr Tyr Leu Asn Ala
                165                 170                 175

Gly Asn Val Phe Phe Asn Ala Met Asn Lys Ala Leu Glu Asn Lys Asn
            180                 185                 190

Gly Thr Ser Ser Ala Ser Gly Thr Ser Gly Ala Thr Gly Ser Asp Gly
        195                 200                 205

Gln Thr Tyr Ser Thr Gln Ala Ile Gln Tyr Leu Gln Gly Gln Gln Asn
    210                 215                 220

Ile Leu Asn Asn Ala Ala Asn Leu Leu Lys Gln Asp Glu Leu Leu Leu
225                 230                 235                 240

Glu Ala Phe Asn Ser Ala Val Ala Ala Asn Ile Gly Asn Lys Glu Phe
                245                 250                 255

Asn Ser Ala Ala Phe Thr Gly Leu Val Gln Gly Ile Ile Asp Gln Ser
            260                 265                 270

Gln Ala Val Tyr Asn Glu Leu Thr Lys Asn Thr Ile Ser Gly Ser Ala
        275                 280                 285

Val Ile Ser Ala Gly Ile Asn Ser Asn Gln Ala Asn Ala Val Gln Gly
    290                 295                 300

Arg Ala Ser Gln Leu Pro Asn Ala Leu Tyr Asn Ala Gln Val Thr Leu
305                 310                 315                 320

Asp Lys Ile Asn Ala Leu Asn Asn Gln Val Arg Ser Met Pro Tyr Leu
                325                 330                 335

Pro Gln Phe Arg Ala Gly Asn Ser Arg Ser Thr Asn Ile Leu Asn Gly
            340                 345                 350

Phe Tyr Thr Lys Ile Gly Tyr Lys Gln Phe Phe Gly Lys Lys Arg Asn
        355                 360                 365

Ile Gly Leu Arg Tyr Tyr Gly Phe Phe Ser Tyr Asn Gly Ala Ser Val
    370                 375                 380

Gly Phe Arg Ser Thr Gln Asn Asn Val Gly Leu Tyr Thr Tyr Gly Val
385                 390                 395                 400

Gly Thr Asp Val Leu Tyr Asn Ile Phe Ser Arg Ser Tyr Gln Asn Arg
                405                 410                 415
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Ser Val Asp Met Gly Phe Phe Ser Gly Ile Gln Leu Ala Gly Glu Thr
420 425 430

Phe Gln Ser Thr Leu Arg Asp Asp Pro Asn Val Lys Leu His Gly Lys
435 440 445

Ile Asn Asn Thr His Phe Gln Phe Leu Phe Asp Phe Gly Met Arg Met
450 455 460

Asn Phe Gly Lys Leu Asp Gly Lys Ser Asn Arg His Asn Gln His Thr
465 470 475 480

Val Glu Phe Gly Val Val Val Pro Thr Ile Tyr Asn Thr Tyr Tyr Lys
485 490 495

Ser Ala Gly Thr Thr Val Lys Tyr Phe Arg Pro Tyr Ser Val Tyr Trp
500 505 510

Ser Tyr Gly Tyr Ser Phe
515

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 656 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:567..656

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGATCTATGA ATCTATGATA TCAACACTCT TTTTGATAAA TTTTCTCGAG GTACCGAGCT
60

TGAGGCATCA AATAAAACGA AAGGCTCAGT CGAAAGACTG GGCCTTTCGT TTTATCTGTT
120

GTTTGTCGGT GAACGCTCTC CTGAGTAGGA CAAATCCGCC GGGAGCGGAT TTGAACGTTG
180

CGAAGCAACG GCCCGGAGGG TGGCGGGCAG GACGCCCGCC ATAAACTGCC ACAAGCTCGG
240

TACCGTTGAT CTTCCTATGG TGCACTCTCA GTACAATCTG CTCTGATGCG CTACGTGACT
300

GGGTCATGGC TGCGCCCCGA CACCCGCCAA CACCCGCTGA CGCGCCCTGA CGGGCTTGTC

360

TGCTCCCGGC ATCCGCTTAC AGACAAGCTG TGACCGTCTC CGGGAGCTGC ATGTGTCAGA
420

GGTTTTCACC GTCATCACCG AAACGCGCGA GGCCCAGCGC TTCGAACTTC TGATAGACTT
480

CGAAATTAAT ACGACTCACT ATAGGGAGAC CACAACGGTT TCCCTCTAGA AATAATTTTG
540

TTTAACTTTA AGAAGGAGAT ATACAT ATG AAA CTG ACT CCC AAA GAG TTA GAC
593
Met Lys Leu Thr Pro Lys Glu Leu Asp
520 525

AAG TTG ATG CTC CAC TAC GCT GGA GAA TTG GCT AAA AAA CGC AAA GAA
641
Lys Leu Met Leu His Tyr Ala Gly Glu Leu Ala Lys Lys Arg Lys Glu
530 535 540

AAA GGC ATT AAG CTT
656
Lys Gly Ile Lys Leu
545

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Lys Leu Thr Pro Lys Glu Leu Asp Lys Leu Met Leu His Tyr Ala
1 5 10 15

Gly Glu Leu Ala Lys Lys Arg Lys Glu Lys Gly Ile Lys Leu
20 25 30

Article 34

New Claims

1. Pharmaceutical composition comprising as an active agent an immunologically protective living vaccine which is a recombinant attenuated bacterium which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable of expressing said nucleic acid molecule or capable of causing the expression of said nucleic acid molecule in a target cell.

Claims

1. ~~Pharmaceutical composition comprising as an active agent an immunologically protective living vaccine which is a recombinant attenuated microbial pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable of expressing said nucleic acid molecule or capable of causing the expression of said nucleic acid molecule in a target cell.~~

2. The composition according to claim 1, wherein the pathogen is an entero-bacterial cell, especially a Salmonella cell.

3. The composition according to claim 1 or 2, wherein the pathogen is a Salmonella aro mutant cell.

4. The composition according to any of claims 1-3, wherein the Helicobacter antigen is urease, a urease subunit, an immunologically reactive fragment thereof, or a peptide mimotope thereof.

5. The composition according to any one of claims 1-3, wherein the Helicobacter antigen is a secretory polypeptide from Helicobacter, an immunologically reactive fragment thereof, or a peptide mimotope thereof.

6. The composition according to any one of claims 1-3 and 5, wherein the Helicobacter antigen is selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive fragments thereof, or a peptide mimotope thereof.

7. The composition according to any one of claims 1-6, wherein said nucleic acid molecule encoding a Helicobacter antigen is capable to be expressed phase variably.

8. The composition according to claim 7,

wherein said nucleic acid molecule encoding the Helicobacter antigen is under control of an expression signal which is substantially inactive in the pathogen and which is capable to be activated by a nucleic acid reorganization caused by a nucleic acid reorganization mechanism in the pathogen.

9. The composition according to claim 8, wherein the expression signal is a bacteriophage promoter, and the activation is caused by a DNA reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

10. The composition according to any one of claims 1-9, wherein said pathogen further comprises at least one second nucleic acid molecule encoding an immunomodulatory polypeptide, wherein said pathogen is capable to express said second nucleic acid molecule.

11. The composition according to any one of claims 1-10, together with pharmaceutically acceptable diluents, carriers and adjuvants.

12. The composition according to claim 11, which is suitable for administration to a mucosal surface or via the parenteral route.

13. A method for the preparation of a living vaccine comprising formulating a pharmaceutical composition according to any one of claims 1-10 in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

14. The method of claim 13 including the preparation of a recombinant attenuated pathogen comprising the steps:

    a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein a recombinant attenuated pathogen is obtained, which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell, and

    b) cultivating said recombinant attenuated pathogen under suitable conditions.

15. The method according to claim 14, wherein said nucleic acid molecule encoding a Helicobacter antigen is located on an extrachromosomal plasmid or inserted in the chromosome.

16. A method for identifying Helicobacter antigens, which raise a protective immune response in a mammalian host, comprising the steps of:

    a) providing an expression gene bank of Helicobacter in an attenuated pathogen and

    b) screening the clones of the gene bank for their ability to confer protective immunity against a Helicobacter infection in a mammalian host.

Figure 1
Genetic map of the expression plasmid pYZ97

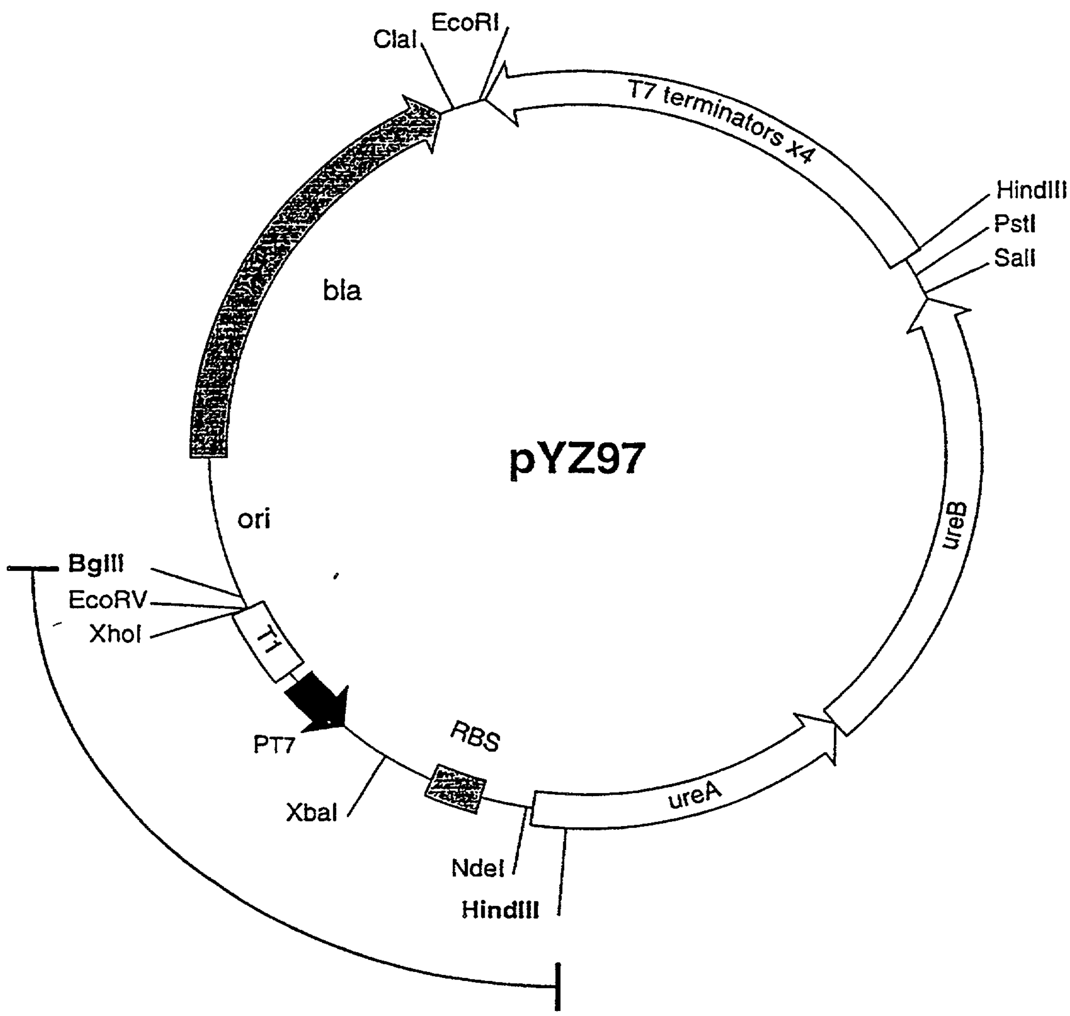

Figure 2

Nucleotide sequence of the transcriptional regulators for urease expression on plasmid pYZ97

```
  1 AG ATC TAT GAA TCT ATG ATA TCA ACA CTC TTT TTG ATA AAT TTT CTC GAG GTA CCG AGC
     BglII               EcoRV                                  XhoI
                                      rrnB T1
 60 TTG AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT

120 TGT TTG TCG GTG AAC GCT CTC CTG AGT AGG ACA AAT CCG CCG GGA GCG GAT TTG AAC GTT
                                                             -35
180 GCG AAG CAA CGG CCC GGA GGG TGG CGG GCA GGA CGC CCG CCA TAA ACT GCC ACA AGC TCG
                -10
240 GTA CCG TTG ATC TTC CTA TGG TGC ACT CTC AGT ACA ATC TGC TCT GAT GCG CTA CGT GAC

300 TGG GTC ATG GCT GCG CCC CGA CAC CCG CCA ACA CCC GCT GAC GCG CCC TGA CGG GCT TGT

360 CTG CTC CCG GCA TCC GCT TAC AGA CAA GCT GTG ACC GTC TCC GGG AGC TGC ATG TGT CAG

420 AGG TTT TCA CCG TCA TCA CCG AAA CGC GCG AGG CCC AGC GCT TCG AAC TTC TGA TAG ACT
                        PT7
480 TCG AAA TTA ATA CGA CTC ACT ATA GGG AGA CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT
                                                             XbaI
                      RBS                      down stream box
                                   YZ019
540 GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AAA CTG ACT CCC AAA GAG TTA GAC AAG TTG
                                        Met Lys Leu Thr Pro Lys Glu Leu Asp Lys Leu

600 ATG CTC CAC TAC GCT GGA GAA TTG GCT AAA AAA CGC AAA GAA AAA GGC ATT AAG CTT
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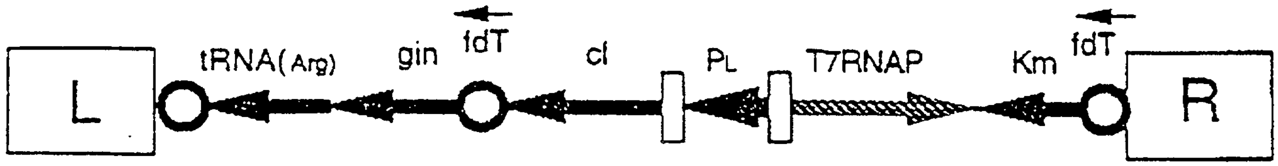
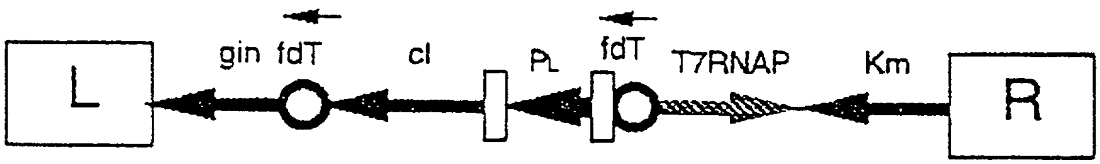
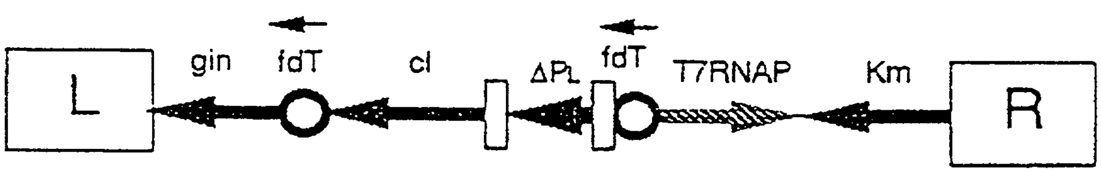
Figure 3



pYZ88 (high expression)



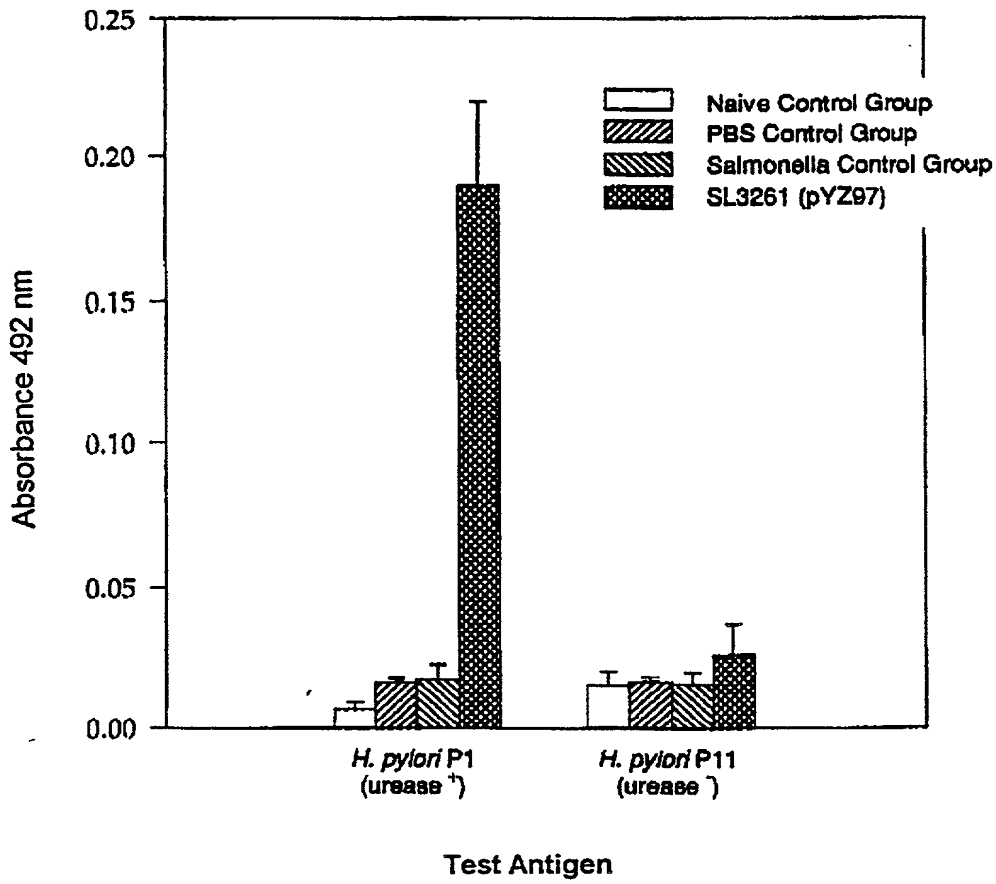
pYZ84 (medium expression)



pYZ114 (low expression)

Figure 4

ELISA for anti-*H. pylori* IgA antibodies in intestinal fluids of vaccinated mice



09/284233

Figure 5

ELISA for anti-*H. pylori* IgA antibodies in serum of vaccinated mice

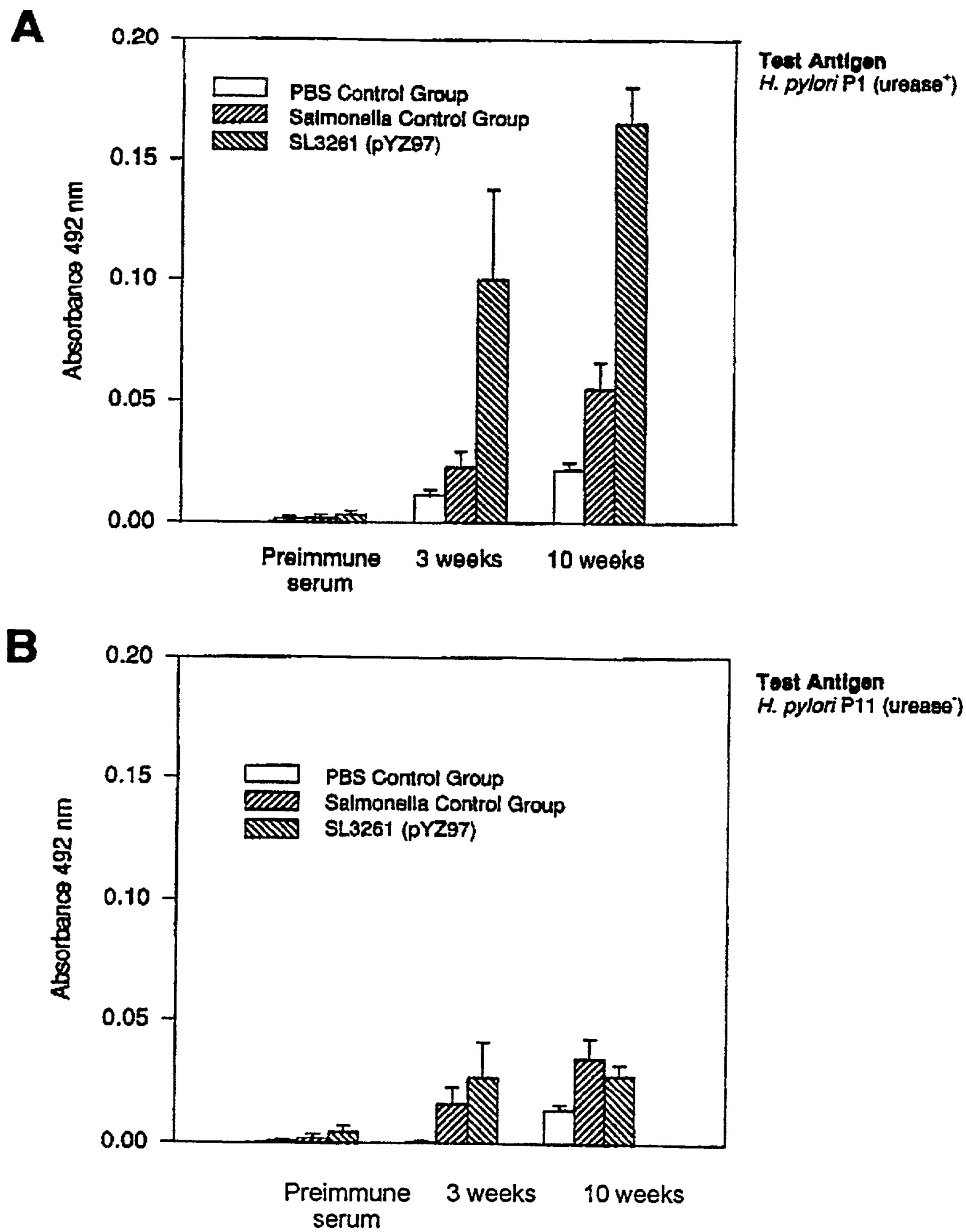
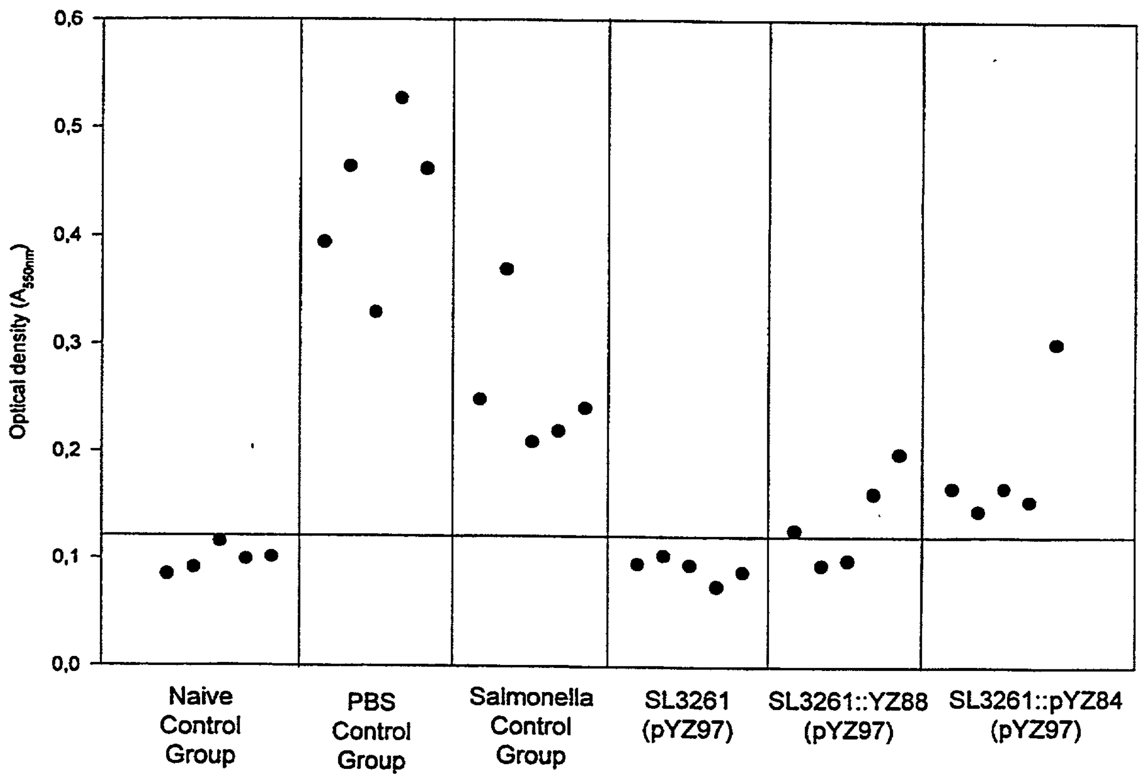


Figure 6

Urease activity in stomach tissue of vaccinated mice after *H. pylori* challenge.



N, M, M & O Docket No. P564-9008 NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below my name.
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) HELICOBACTER PYLORI LIVE VACCINE
the specification of which is attached hereto unless the following box is checked:

☒ was filed on September 1, 1997 as PCT International Application Number PCT/EP97/04744 and was amended on ________

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.
I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications-See note A on back of this page)				Priority Claimed
	96116337.5 (Number)	EP (Country)	11 October 1996 (Day/Month/Year Filed)	☒ Yes ☐ No
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)			
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys: David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; E. Marcie Emas, Reg. No. 32,131; Douglas H Goldhush, Reg. No. 33,125; Monica Chin Kitts, Reg. No. 36,105; Richard J. Berman, Reg. No. 39,107; King L. Wong, Reg. No. 37,500; Karen K. Costantino, Reg. No 35,107; James A. Poulos, III, Reg. No. 31,714; Herbert C. Rose, Reg. No. 29,846; and Patrick D. Muir, Reg. No. 37,403.

(13)

Please direct all communications to the following address: NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor: Thomas F. MEYER

Inventor's signature X ________ X ________ Date

Residence: Spemannstrasse 30, D-72076 Tübingen, Germany

Citizenship: German

Post Office Address: Same as above

Rev. #13 2/22/99

N, M, M & O Docket No. P564-9008 NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below my name.
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled (Insert Title) HELICOBACTER PYLORI LIVE VACCINE
the specification of which is attached hereto unless the following box is checked:

☒ was filed on September 1, 1997 as PCT International Application Number PCT/EP97/04744 and was amended on ______________

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.
I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications. See note A on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
	96116337.1	EP	11 October 1996	☒ Yes ☐ No
				☐ Yes ☐ No
				☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys: David T. Nikaido, Reg. No. 22,663, Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980, E. Marcie Emas, Reg. No. 32,131, Douglas H. Goldhush, Reg. No. 33,125; Monica Chin Kitts, Reg. No. 36,105, Richard J. Berman, Reg. No. 39,107; King L. Wong, Reg. No. 37,500; Karen K. Costantino, Reg. No. 35,107; James A. Poulos, III, Reg. No. 31,714, Herbert C. Rose, Reg. No. 29,346, and Patrick D. Muir, Reg. No. 37,403

Second page added

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(202) 638-5000 Fax. (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

(See Note C on back of this page)

Full name of sole or first inventor: Thomas F. MEYER
Inventor's signature: X [signature] 22.6.99
Date
Residence: Spemannstrasse 30, D-72076 Tübingen, Germany DEX
Citizenship: German
Post Office Address: Same as above

Rev #13 2/22/99

2-00

Full name of second joint inventor, if any: Rainer HAAS

Inventor's signature X Rainer Haas X 09/06/99

Date

Residence: ~~[illegible]~~ WENINGSTR. 12, D-81547 MÜNCHEN GERMANY DEX

Citizenship: German

Post Office Address: Same as above

R. Haas

Full name of third joint inventor, if any: Yan ZHENGXIN

Inventor's signature X X

Date

Residence: Fichtenweg 6, D-72076, Tübingen, Germany

Citizenship: Chinese

Post Office Address: Same as above

4-00

Full name of fourth joint inventor, if any: Oscar GOMEZ-DUARTE

Inventor's signature X Oscar Gomez X 04-07-99

Date

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5-00

Full name of fifth joint inventor, if any: Bernadette LUCAS

Inventor's signature X Lucas X 16/06/99

Date

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Lucas

Full name of second joint inventor, if any: Rainer HAAS

Inventor's signature X ________________ X ________ Date

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3-00

Full name of third joint inventor, if any: ~~Yan ZHENGXIN~~ Zhengxin YAN X 10.06.1999

Inventor's signature X ________________ Date

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Full name of fourth joint inventor, if any: Oscar GOMEZ-DUARTE

Inventor's signature X ________________ X ________ Date

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Full name of fifth joint inventor, if any: Bernadette LUCAS

Inventor's signature X ________________ X ________ Date

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